

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property
Organization
International Bureau



(43) International Publication Date
3 June 2004 (03.06.2004)

PCT

(10) International Publication Number
WO 2004/046346 A2

(51) International Patent Classification⁷: C12N 1/00 (74) Common Representative: VIB VZW; Rijvisschestraat 120, B-9052 Zwijnaarde (BE).

(21) International Application Number:

PCT/EP2003/050832

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(22) International Filing Date:

14 November 2003 (14.11.2003)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

02079786.6 15 November 2002 (15.11.2002) EP

(84) Designated States (*regional*): ARIPO patent (BW, GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

(71) Applicants (*for all designated States except US*): VIB VZW [BE/BE]; Rijvisschestraat 120, B-9052 Zwijnaarde (BE). UNIVERSITEIT GENT [BE/BE]; Sint-Pietersnieuwstraat 25, B-9000 Gent (BE).

Published:

— without international search report and to be republished upon receipt of that report

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(72) Inventors; and

(75) Inventors/Applicants (*for US only*): STEIDLER, Lothar [BE/IE]; Finnis, Bandon, Co Cork (IE). ROT-TIERS, Pieter [BE/BE]; Twee Drenen 37, B-9840 De Pinte (BE). REMAUT, Erik [BE/BE]; Bergstraat 7, B-9921 Vinderhoute (BE).



WO 2004/046346 A2

(54) Title: SELF-CONTAINING LACTOBACILLUS STRAIN

(57) Abstract: The invention relates to a recombinant Lactobacillus strain, with limited growth and viability in the environment. More particularly, it relates to a recombinant Lactobacillus that can only survive in a medium, where well-defined medium compounds, preferably thymidine or thymine, are present. A preferred embodiment is a Lactobacillus that may only survive in a host organism, where said medium compounds are present, but cannot survive outside the host organism in absence of said medium compounds. Moreover, said Lactobacillus strain can be transformed with prophylactic and/or therapeutic molecules and can, as such, be used to treat diseases such as, but not limited to, inflammatory bowel diseases.

SELF-CONTAINING *Lactobacillus* STRAIN**Field of the invention**

The invention relates to a recombinant *Lactobacillus* strain, with limited growth and viability in
5 the environment. More particularly, it relates to a recombinant *Lactobacillus* that can only survive in a medium, where well-defined medium compounds, preferably thymidine or thymine, are present. A preferred embodiment is a *Lactobacillus* that may only survive in a host organism, where said medium compounds are present, but cannot survive outside the host organism in absence of said medium compounds. Moreover, said *Lactobacillus* strain can be
10 transformed with prophylactic and/or therapeutic molecules and can, as such, be used to treat diseases such as, but not limited to, inflammatory bowel diseases.

Background of the invention

Lactic acid bacteria have long time been used in a wide variety of industrial fermentation processes. They have generally-regarded-as-safe status, making them potentially useful organisms for the production of commercially important proteins. Indeed, several heterologous proteins, such as Interleukin-2, have been successfully produced in *Lactococcus* spp (Steidler *et al.*, 1995). It is, however, unwanted that such genetically modified micro organisms are surviving and spreading in the environment.
20 To avoid unintentional release of genetically modified microorganisms, special guidelines for safe handling and technical requirements for physical containment are used. Although this may be useful in industrial fermentations, the physical containment is generally not considered as sufficient, and additional biological containment measures are taken to reduce the possibility of survival of the genetically modified microorganism in the environment. Biological containment
25 is extremely important in cases where physical containment is difficult or even not applicable. This is, amongst others, the case in applications where genetically modified microorganisms are used as live vaccines or as vehicle for delivery of therapeutic compounds. Such applications have been described e.g. in WO 97/14806, which discloses the delivery of biologically active peptides, such as cytokines, to a subject, by recombinant non-invasive or
30 non-pathogenic bacteria. WO 96/11277 describes the delivery of therapeutic compounds to an animal – including humans - by administration of a recombinant bacterium, encoding the therapeutic protein. Steidler *et al.* (2000) describe the treatment of colitis by administration of a recombinant *Lactococcus lactis*, secreting interleukin-10. Such a delivery may indeed be extremely useful to treat a disease in an affected human or animal, but the recombinant
35 bacterium may act as a harmful and pathogenic micro organism when it enters a non-affected subject, and an efficient biological containment that avoids such unintentional spreading of the micro organism is needed.

Although a sufficient treatment can be obtained using *Lactococcus*, it has as main disadvantage that the bacterium is not colonizing and that the medication should applied in a continuous way, to ensure the effect. A colonizing strain like *Lactobacillus* would have the advantage that a similar effect can be used with a single dose or a limited number doses.

- 5 However, similar to the lactobacillus case, a stringent biological containment system is needed to avoid the dissemination of the bacterium in the environment.

Biological containment systems for host organisms may be passive, based on a strict requirement of the host for specific growth factor or a nutrient, that is not present or present in low concentrations in the outside environment, or active, based on so-called suicidal genetic elements in the host, whereby the host is killed in the outside environment by a cell killing function, encoded by a gene that is under control of a promoter only being expressed under specific environmental conditions.

Passive biological containment systems are well known in microorganisms such as *Escherichia coli* or *Saccharomyces cerevisiae*. Such *E. coli* strains are disclosed e.g. in 5 US4100495. WO 95/10621 discloses lactic acid bacterial suppressor mutants and their use as means of containment in lactic acid bacteria, but in that case, the containment is on the level of the plasmid, rather than on the level of the host strain and it stabilizes the plasmid in the host strain, but doesn't provide containment for the genetically modified host strain itself. A similar containment system on the level of the plasmid has been described for *Lactobacillus* 0 *acidophilus* by Fu and Xu (2000), using the *thyA* gene from *Lactobacillus casei* as selective marker. The *thyA* mutant used has been selected by spontaneous mutagenesis and trimethoprim selection. Such a mutation is prone to reversion and the *thyA* gene of another *Lactobacillus* species is used to avoid the reversion of the mutation by recombination of the marker gene. Indeed, reversion of the *thyA* mutation is a problem, and especially in absence of 5 thymine or thymidine in the medium, the mutation will revert at high frequency, whereby the strain is losing its containment characteristics. For an acceptable biological containment, a non-reverting mutant is wanted.

Non-reverting mutants can be obtained by gene disruption. However, although the *thyA* gene of *Lactobacillus casei* has been mutated by site directed mutagenesis, it was only tested in *E. coli*, and never used for gene replacement in a *Lactobacillus* strain. Although transformation techniques for *Lactobacillus* are known to the person skilled in the art, gene disruption of *thyA* in *Lactobacillus* has never succeeded and is clearly not evident.

Active suicidal systems have been described by several authors. Such system consists of two elements: a lethal gene, and a control sequence that switches on the expression of the lethal 5 gene under non-permissive conditions. WO 95/10614 discloses the use of a cytoplasmatically active truncated and/or mutated *Staphylococcus aureus* nuclease as lethal gene. WO 96/40947 discloses a recombinant bacterial system with environmentally limited viability,

based on the expression of either an essential gene, expressed when the cell is in the permissive environment and is not expressed or temporarily expressed when the cell is in the non-permissive environment and/or a lethal gene, wherein expression of the gene is lethal to the cell and the lethal gene is expressed when the cell is in the non-permissive environment
5 but not when the cell is in the permissive environment. WO 99/58652 describes a biological containment system based on the *relE* cytotoxin. However, most systems have been elaborated for *Escherichia coli* (Tedin et al., 1995; Knudsen et al., 1995; Schweder et al., 1995) or for *Pseudomonas* (Kaplan et al., 1999; Molina et al., 1998). Although several of the containment systems theoretically can be applied to lactic acid bacteria, no specific biological
0 containment system for *Lactobacillus* has been described that allows the usage of a self-containing and transformed *Lactobacillus* to deliver prophylactic and/or therapeutic molecules in order to prevent and/or treat diseases.

Description of the Invention

5 It is the objective of the present invention to provide a suitable biological containment system for *Lactobacillus*.
A first aspect of the invention is an isolated strain of *Lactobacillus* sp. comprising a mutant thymidylate synthase gene (*thyA*), whereby said gene is inactivated by gene disruption. Gene disruption, as used here, includes disruption insertion of a DNA fragment, disruption by
0 deletion of the gene, or a part thereof, as well exchange of the gene or a part thereof by another DNA fragment. Preferably, disruption is the exchange of the gene, or a part thereof, by another functional gene. Preferably, said mutant thymidylate synthase is a non-reverting mutant.
A non-reverting mutant as used here means that the reversion frequency is lower than 10^{-8} ,
5 preferably the reversion frequency is lower than 10^{-10} , even more preferably, said reversion frequency is lower than 10^{-12} , even more preferably, said reversion frequency is lower than 10^{-14} , most preferably, said reversion frequency is not detectable using the routine methods known to the person skilled in the art. Preferably, said *Lactobacillus* sp. is *Lactobacillus salivarius* or *Lactobacillus plantarum*. A non-reverting *thyA* mutant strain can be considered as
0 a form of active containment, as it will undergo cell death in response to thymine and thymidine starvation (Ahmad et al., 1998).

The *Lactobacillus casei* thymidylate synthase gene has been cloned by Pinter et al. (1988). CN1182134 discloses a vector devoid of antibiotic resistance and bearing a thymidylate synthase gene as a selection marker; the same vector has been described by Fu and Xu
5 (2000) for *Lactobacillus acidophilus*. However, in this specific case, reversion of the mutation is prevented by complementing the mutation by the *L. casei* gene, that shows only a low homology; the stability of the mutation is only guaranteed in presence of the complementing

vector, or when thymine or thymidine is supplied to the medium. The mutant strain may not be stable enough to use in medical situations where a strict biological containment is needed. The present invention discloses how to construct such mutant by gene disruption, using homologous recombination in *Lactobacillus*.

- 5 In a preferred embodiment, the *thyA* gene of a *Lactobacillus* sp. strain, preferably *Lactobacillus salivarius* or *Lactobacillus plantarum*, is disrupted and replaced by a functional human interleukin-10 expression cassette. Said interleukin-10 expression unit is preferably, but not limited to, a human interleukin-10 expression unit or gene encoding for human interleukin-10. However, it is clear that any construct can be used for gene disruption, as long as it results in
0 an inactivation of the *thyA* gene or in an inactive thymidylate synthase. As a non-limiting example, the homologous recombination may result in a deletion of the gene, in one or more amino acid substitutions that lead to an inactive form of the thymidylate synthase, or to a frameshift mutation resulting in a truncated form of the protein.

Another aspect of the invention is the use of a strain according to the invention as host strain

- 5 for transformation, whereby the transforming plasmid does not comprise an intact thymidylate synthase gene. Such a *Lactobacillus* sp. *thyA* mutant is very useful as a host strain in situations where more severe containment than purely physical containment is needed. Indeed, *thyA* mutants cannot survive in an environment without, or with only a limited concentration of thymidine and/or thymine. When such a strain is transformed with a plasmid that doesn't
0 comprise an intact *thyA* gene and cannot complement the mutation, the transformed strain will become suicidal in a thymidine/thymine poor environment. Such a strain can be used in a fermentor, as an additional protection for the physical containment. Moreover, the present invention discloses that such a strain is especially useful in cases where the strain is used as a delivery vehicle in an animal body, including the human body. Indeed, when such a
5 transformed strain is given for example orally to an animal – including humans – it survives in the gut, and produces homologous and/or heterologous proteins, such as human interleukin-10, that may be beneficial for said animal.

Still another aspect of the invention is a transformed strain of *Lactobacillus* sp. according to the invention, comprising a plasmid that does not comprise an intact thymidylate synthase gene.

- 0 The transforming plasmid can be any plasmid, as long as it cannot complement the *thyA* mutation. It may be a selfreplicating plasmid that preferably carries one or more genes of interest and one or more resistance markers, or it may be an integrative plasmid. In the latter case, a special case of transformation is the one whereby the integrative plasmid itself is used
5 to create the *thyA* mutation, by causing integration at the *thyA* site, whereby the *thyA* gene is inactivated. Preferably, the active *thyA* gene is replaced by double homologous recombination by a cassette comprising the gene or genes of interest, flanked by targeting sequences that target the insertion to the *thyA* target site. In this case, the introduction of the mutation and the

- transformation with the gene of interest is carried out in one and the same transformation experiment. It is of extreme importance that these targeting sequences are sufficiently long and sufficiently homologous to obtain integration of the sequence into the target site. However, to avoid the problem of the long homologous sequences, a recombinase assisted cross over
- 5 may be used. Transformation methods of *Lactobacillus* are known to the person skilled in the art, and include, but are not limited to protoplast transformation and electroporation.
- Another aspect of the invention relates to a transformed strain of *Lactobacillus* sp. comprising a gene or expression unit encoding a prophylactic and/or therapeutic molecule. Preferably, said prophylactic and/or therapeutic molecule is interleukin-10.
- 0 Consequently, the present invention also relates to the usage of a transformed strain of *Lactobacillus* sp. to deliver prophylactic and/or therapeutic molecules, and as such, to treat diseases. The delivery of such molecules has been disclosed, as a non-limiting example, in WO 97/14806 and in WO 98/31786. Prophylactic and/or therapeutical molecules include, but are not limited to polypeptides such as insulin, growth hormone, prolactine, calcitonin, group 1
- 5 cytokines, group 2 cytokines and group 3 cytokines and polysaccharides such as polysaccharide antigens from pathogenic bacteria. A preferred embodiment is the use of a *Lactobacillus* sp. strain according to the invention to deliver human interleukin-10. Methods to deliver said molecules and methods to treat diseases such as inflammatory bowel diseases are explained in detail in WO 97/14806 and WO 00/23471 to Steidler et al. and in Steidler et al.
- 0 (2000) that are hereby incorporated by reference. The present invention demonstrates that the strain according to the invention surprisingly passes the gut at the same speed as the control strains and shows that their loss of viability is indeed not different from that of the control strains. However, once said strain is secreted in the environment, e.g. in the faeces, it is not able to survive any longer. The fact that the deletion mutant can survive in the intestine, and
- 5 more specifically in the ileum, and as such can be used as a biologically contained delivery strain is especially surprising, as it is known that the dependency upon thymine by the known *thyA* mutants is rather high (about 20 μ g/ml; Ahmad et al., 1998); based on this data, one would expect that mutant can't survive in the ileum where there is only a very limited concentration of thymine present.
- 0 Another aspect of the invention is a pharmaceutical composition, comprising a *Lactobacillus* sp. *thyA* disruption mutant, according to the invention. As a non-limiting example, the bacteria may be encapsulated to improve the delivery to the intestine. Methods for encapsulation are known to the person, skilled in the art, and are disclosed, amongst others, in EP0450176.
- Still another aspect of the invention is the use of a strain according to the invention for the
- 5 preparation of a medicament. Preferably, said medicament is used to treat Crohn's disease or inflammatory bowel disease.

Brief description of the figures

- Figure 1:** plasmid map of the pKD46 plasmid that upon arabinose induction expresses the phage λ Red recombinases. Bla, ampicillin resistance. gam, γ gene. bet, β gene. exo, exo gene. P_{araB} , arabinose-inducible promoter.
- 5 **Figure 2:** Plasmid map of ORI⁺ RepA⁻ pORI19. lacZ, lacZ α fragment from pUC19. Em, erythromycin resistance gene. Only relevant restriction enzyme sites are shown.
- Figure 3:** Construction schedule of the vector pORI-RED.
- 10 **Figure 4:** System of gene-replacement of the *Lactobacillus thyA* gene by hIL-10 with the aid of the lambda red recombinases

Examples

- Example 1: general outline of the experiment**
- On the base of the *Lactobacillus casei* or the *Lactobacillus plantarum* sequence, the Thy A gene is localized in *L. salivarius*, or any other suitable *Lactobacillus* species. Starting from this sequence, the sequences adjacent to the Thy A gene are cloned and sequenced.
- 5 The knowledge of these sequences is of critical importance for the genetic engineering of any *Lactobacillus* strain in a way as described below, as the strategy will employ double homologous recombination in the areas 1000 bp at the 5'end and 1000 bp at the 3'end of *thyA*, the "thyA target". These sequences are not available from any public source to date. We have
- 10 cloned these flanking DNA fragments and have identified their sequence.
- The *thyA* replacement is performed by homologous recombination, essentially as described by Biwas et al. (1993). Suitable replacements in a plasmid borne version of the *thyA* target are made, as described below. The carrier plasmid is a replication defective plasmid, which only transfers the erythromycin resistance to a given strain when a first homologous recombination,
- 15 at either the 5' 1000bp or at the 3' 1000bp of the *thyA* target. A second homologous recombination at the 3' 1000bp or at the 5' 1000bp of the *thyA* target yields the desired strain.
- Alternatively, a recombinase assisted inrecombination may be used. This allows the use of shorter 5' and 3' sequences.
- 20 The *thyA* gene is replaced by a synthetic gene encoding a protein which has a secretion leader, functional in *Lactobacillus* fused to a protein of identical amino acid sequence than: (a) the mature part of human-interleukin 10 (hIL-10) or (b) the mature part of hIL-10 in which proline at position 2 had been replaced with alanine.
- The resulting strains are *thyA* deficient, a mutant not yet described for *L. salivarius*. It is strictly dependent upon the addition of thymine or thymidine for growth.
- 25 5 The region around the inserted hIL-10 gene is isolated by PCR and the DNA sequence is verified. The structure is identical to the predicted sequence.

Human interleukin-10 production in the mutants is checked by western blot analysis, and compared with the parental strain, transformed with an empty plasmid as negative control, and the parental strain, transformed with the IL10 producing plasmid as positive control. The concentration in the culture supernatant is quantified using ELISA. All isolates of the mutant
5 produce a comparable, significant amount of hIL-10, be it less than the strain, transformed with the non-integrative plasmid.

Quantification of hIL-10 present in the culture supernatant of the indicated strains is done by ELISA. The N-terminal protein sequence of the recombinant hIL-10 is determined by Edman degradation and is shown identical to the structure as predicted for the mature, recombinant
10 hIL-10. The protein shows full biological activity.

The effect of the thymidylate synthase deletion on the growth in thymidine less and thymidine supplemented media is tested. Absence of thymidine in the medium strongly limits the growth of the mutant, and even results in a decrease of colony forming units after four hours of cultivation in absence of thymidine or thymine. Addition of thymidine to the medium results in
15 an identical growth curve and amount of colony forming units, compared to the wild type strain, indicating that the mutant doesn't affect the growth or viability in thymidine supplemented medium.

Mouse experiments are carried out, proving that the *Lactobacillus salivarius* thyA mutant is able to survive in the ileum of the mice, but can't survive outside the intestine. The colony
!0 count of the mutant in the faeces drops dramatically, when compared to the wild type strain, indicating that the strain is a useful tool for delivery under in the intestine under conditions of biological containment.

Example 2: Identification of the thymidylate synthase (*thyA*) regio in *Lactobacillus* species.

!5 Based on the publication of Kleerebezem *et al.*, 2003, we had web-based access to the complete genome sequence of *Lactobacillus plantarum* WCFS1. Based on a blastn between the complete genome of the *Lactobacillus plantarum* WCFS1 and the *thyA* gene of *E. coli* K12, we identified the *thyA* gene in *Lactobacillus*.

Based on these published *thyA* DNA sequence of *Lactobacillus plantarum* WCFS1
!0 degenerate oligonucleotides are synthesized to be used as primers for DNA sequencing of the *thyA* gene of any particular *Lactobacillus* species. Once the sequence of the *thyA* gene of that particular *Lactobacillus* species is known, oligonucleotides are designed as primers for DNA-sequencing of the 5' and 3' flanking regions of the *thyA* gene. The identification of the 5' and 3' flanking regions (a stretch of 50 nucleotides upstream and downstream of the *thyA* gene is

sufficient) is necessary for the gene-replacement of the *thyA* gene by the *human interleukine-10* gene (*hIL-10* gene).

Example 3: gene-replacement of the *thyA* gene by the *hIL-10* gene

The system of gene-replacement that is used in *Lactobacillus* is an adaptation of a system introduced by Datsenko et al. (2000). This is a simple and highly efficient method to disrupt chromosomal genes in *Escherichia coli*. In this procedure, PCR primers provide the homology to the targeted gene(s) and recombination depends on the phage λ Red recombinases, which are synthesized under the control of an arabinose-inducible promoter on an easily curable, low copy number plasmid, plasmid pKD46 (Fig. 1). This recombination pathway not only ensures that, after electroporation of the linear PCR fragment into the cell, the linear DNA is not instantly degraded, but it allows also an efficient gene-replacement by a double cross-over with a limited homology of only 36- to 50-nucleotides to the regions adjacent to the gene that need to be replaced.

The pKD46 plasmid is an *E. coli* plasmid. To adapt this method to *Lactobacillus*, it is necessary that the λ Red recombinases are subcloned into a plasmid that can replicate in *Lactobacillus*. The λ Red recombinase operon is subcloned in the broad host shuttle vector pORI19 (Fig. 2; Law et al., 1995). pORI19 is preferred because it is based on the conditional replicon of the lactococcal pWV01-derived Ori⁺ RepA⁻ vector. Due to the fact that the pORI19 is missing the *repA* gene, it is replication deficient. For the replication of the pORI19 plasmid, the helper plasmid pVE6007 (Maguin et al., 1992) needs to provide the RepA-Ts protein in *trans*. The replication of the helper plasmid pVE6007 is temperature sensitive. A temperature of 30°C is permissive for the replication of the plasmid, while a temperature shift to 37 °C abolishes its replication and induces the loss of the plasmid. The loss of the helper plasmid pVE6007 results in the loss of the pORI19 plasmid. Assembly of pORI19-derived plasmids is carried out in the *E. coli* helper strain EC101, which has the *repA* gene genomically integrated.

Construction of pORI-RED

pORI-RED is the pORI19 plasmid in which the λ Red recombinase operon from the vector pKD46 is subcloned under control of the arabinose inducible promotor. All the constructs are made in the *E. coli* helper strain EC101.

0 By use of PCR the λ Red recombinase operon is amplified (Fig. 3). The primers of the PCR are designed in such a way that a *Pvu*I site is introduced at the 5' end of the operon and an *Xba*I site is introduced at the 3' end. This PCR-fragment is cut by a combined digestion of *Pvu*I and *Xba*I and ligated in the by *Pvu*I and *Xba*I linearized pORI19 vector. This ligated plasmid is electroporated to the *E. coli* helper strain EC101 (for construction scheme, Fig. 3)

*Preparation of the recombination ready *Lactobacillus* cells.*

Prior to gene-replacement of the *thyA* gene by *hil-10*, we prepare competent cells of the *Lactobacillus* strain and introduce the plasmids, pVE6007 and pORI-RED, by electroporation. Because of the temperature sensitivity of the plasmid pVE6007, all manipulations are
5 conducted at 30°C. The introduction of these two plasmids in the *Lactobacillus* species is done in two steps. In the first step the plasmid pVE6007 is electroporated in the electrocompetent *Lactobacillus* strain. Chloramphenicol is added to the medium to ensure the stability of pVE6007. The resulting *Lactobacillus* strain is made electrocompetent again and the plasmid pORI-RED is electroporated in this *Lactobacillus* strain, using erythromycin as
0 selectable marker. The resulting *Lactobacillus* strain harbouring pVE6007 and pORI-RED is made electrocompetent by an adapted protocol. Thereto, an overnight *Lactobacillus* culture is 1/100 diluted in 250 ml MRS (Difco) + erythromycin and chloramphenicol, and 1 mM L-arabinose added. This ensures that the arabinose promotor of the pORI-RED plasmid is activated and that the three λ Red recombinases are expressed which makes recombination
5 possible in the next step.

Generation of the gene-replacement PCR fragment.

As described in figure 4, a linear PCR fragment is used for the gene-replacement of the genomic *thyA* gene by the *hil-10* gene. For the PCR reaction, primers with 36- to 50-
0 nucleotide extensions homologous to regions adjacent to the genomic *thyA* gene are used,
and a plasmid that carries the *hil-10* is used as template. This PCR carried out on the template plasmid pT1hil10 with the sense primer 5' *thyA* and the antisense primer 3' *thyA* (Figure 4, STEP 1). The resulting PCR product is cleaned up with the Qiagen Qiaquick PCR
purification kit (cat# 28104). This purified PCR product is digested by *Dpn*I for one hour to
5 remove residual template (the plasmid pT1hil10). Afterwards the PCR product is fenol/chloroform extracted and precipitated by ethanol with the aid of seeDNA (Amersham biotech, cat# RPN 5200). The resulting PCR product pellet is dissolved in 5 µl TE buffer (Tris-EDTA).

*Electroporation of the PCR fragment into *Lactobacillus**

0 The PCR fragment that was generated in STEP 1, together with a selection plasmid, are now electroporated in the electrocompetent *Lactobacillus* strain containing the plasmids pVE6007 and pORI-RED. The 5 µl PCR mixture and the selection plasmid are mixed with 100 µl electrocompetent *Lactobacillus* cells. The cells are electroporated with a Biorad genepulser II using the following conditions: 50 µF, 1.7 kV, 200 Ω whereafter 1 ml MRS + 50 µg/ml

- thymidine is added to the cells. This *Lactobacillus* cell mixture is kept for 2 hours at 37°C. These 2 hours allow gene-replacement of the genomic *Lactobacillus thyA* gene by the *hIL-10* gene with the aid of the λ Red recombinases. By growing the cells at 37°C, the plasmid pVE6007 is inhibited in his replication and is lost, resulting in the subsequent loss of pORI-Red.
- 5 After the two hours of incubation at 37°C the *Lactobacillus* suspension is plated out at 30°C on 3 MRS plates (350 µl per plate) containing 50 µg/ml thymidine and the antibiotic for which the selection plasmid specifies resistance. This step eliminates those cells in the electroporation mixture that were not competent for DNA uptake and provides a considerable enrichment for progeny cells derived from the fraction of competent cells that have taken up
- 10 the selection plasmid. These have a high probability of also having taken up the linear PCR fragment generated in STEP1.

Example 4: Identification of a *thyA*⁻ and *IL-10*⁺ *Lactobacillus****Primary thyA*⁻ and *IL-10*⁺ selection by PCR**

- 5 The primary screening of the *Lactobacillus* colonies carrying a *hIL-10* insert is done by colony PCR screening. A small part of each *Lactobacillus* colony is added to the respectively PCR master mix. Two different PCR screenings are conducted on each *Lactobacillus* colony. The first PCR screening is the one where the primers are indicated by 1 and 2 on figure 4, STEP 2. In the negative colonies (no PCR product) the *thyA* gene is removed from the *Lactobacillus* genome and *Lactobacillus* strain is *thyA* negative. The second PCR screening is one with the primers 1 and 3 on figure 4, STEP 3. Positive colonies (a PCR product of approximately 1000 bp) are isolated. In these colonies, the *Lactobacillus* strain carries a genetically integrated copy of the *hIL-10* gene.

Confirmation of the *thyA*⁻ and *IL-10*⁺ properties of the *Lactobacillus* by Southern blot.

- 5 From the positive *Lactobacillus* colonies, a genomic DNA preparation is made. The genomic *Lactobacillus* DNA is digested by *Sph*I and *Nde*I and Southern blotted. The blot is revealed with digoxigenin-labeled probes for identifying *thyA* (*thyA* probe) or *hIL-10* (*hIL-10* probe). As expected on base of the PCR results, the *thyA* probe signal is negative and the *hIL-10* probe signal on the blot is positive.

0

Example 5: Production of human IL-10 by the *thyA*⁻ and *IL-10*⁺ *Lactobacillus*

To evaluate the *hIL-10* secretion, the strain is grown in buffered minimal medium (BM9) that contains 50 µg/ml thymidine. After 12 hours of growth at 37 °C of 4 x 10⁷ cells, the medium is

tested for the prevalence of human IL-10 by Western blot and ELISA. The *Lactobacillus* strain is secreting a sufficient amount of human IL-10 in the culture supernatant to be used in *in vivo* experiments.

5 Example 6: Curing of resident plasmids

For use in *in vivo* experiments the thyA⁻ and IL-10⁺ *Lactobacillus* strain is preferably free of any resident plasmid. This can be accomplished by successive rounds of curing (reviewed in: de Vos, 1987).

References

- Ahmad, S.I., Kirk, S.H. and Eisenstark, A. (1998) Thymine metabolism and thymineless death in prokaryotes and eukaryotes. *Annu. Rev. Microbiol.* **52**, 591 – 625.
- Biswas, I., Gruss, A., Ehrlich, S.D. et al. (1993) High-efficiency gene inactivation and replacement system for gram- positive bacteria. *J. Bacteriol.* **175**, 3628 – 3635.
- De Vos, W.M. (1987) Gene cloning and expression in lactic streptococci. *GEMS Microbiol. Rev.* **46**, 281-295.
- Datsenko KA, Wanner BL. One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. *Proc Natl Acad Sci U S A* 2000; **97**:6640-6645.
- 0 - Fu, X. and Xu, J.G. (2000) Development of a chromosome-plasmid balanced lethal system for *Lactobacillus acidophilus* with ThyA gene as selective marker.
- Gasson, M. J. (1983). Plasmid complements of *Streptococcus lactis* NCDO 712 and other lactic streptococci after protoplast-induced curing. *J. Bacteriol.* **154**, 1 – 9.
- Kaplan, D.L., Mello, C., Sano, T., Cantor, C. and Smith, C. (1999). Streptavidin-based containment system for genetically engineered microorganisms. *Biomol. Eng.* **31**, 135 – 140.
- 5 - Kleerebezem M, Boekhorst J, van Kranenburg R, Molenaar D, Kuipers OP, Leer R, Tarchini R, Peters SA, Sandbrink HM, Fiers MW, Stiekema W, Lankhorst RM, Bron PA, Hoffer SM, Groot MN, Kerkhoven R, de Vries M, Ursing B, de Vos WM, Siezen RJ. Complete genome sequence of *Lactobacillus plantarum* WCFS1. *Proc Natl Acad Sci U S A* 2003; **100**:1990-1995.
- 0 - Knudsen, S., Saadbye, P., Hansen, L.H., Collier, A., Jacobsen, B.L., Schlundt, J. And Karlstrom, O.H. (1995). Development and testing of improved suicide functions for biological containment of bacteria. *Appl. Environ. Microbiol.* **61**, 985 – 991.
- 5 - Law J, Buist G, Haandrikman A, Kok J, Venema G, Leenhouts K. A system to generate chromosomal mutations in *Lactococcus lactis* which allows fast analysis of targeted genes. *J. Bacteriol.* 1995. **177**:7011-7018
- Molina, L., Ramos, C., Ronchel, M.C., Molin, S. and Ramos, J.L. (1998). Construction of an efficient biologically contained *pseudomonas putida* strain and its survival in outdoor assays. *Appl. Environ. Microbiol.* **64**, 2072 – 2078.
- 0 - Pinter, K., Davisson, V.J. and Santi, D.V. (1988). Cloning, sequencing, and expression of the *Lactobacillus casei* thymidine synthase. *DNA* **7**, 235-241.
- Schweder, T., Hofmann, K. And Hecker, M. (1995). *Escherichia coli* K12 *relA* strains as safe hosts for expression of recombinant DNA. *Appl. Environ. Microbiol.* **42**, 718 – 723.
- 5 - Steidler, L., Hans, W., Schotte, L., Neirynck, S., Obermeier, F., Falk, W., Fier, W. and Remaut, E. (2000). Treatment of murine colitis by *Lactococcus lactis* secreting Interleukin-10. *Science* **289**, 1352 – 1355.

- Steidler, L., Wells, J.M., Raeymaekers, A., Vandekerckhove, J., Fiers, W. And Remaut, E. (1995). Secretion of biologically active murine Interleukin-2 by *Lactococcus lactis* subsp. *Lactis*. *Appl. Environ. Microbiol.* **61**, 1627-1629.
- Tedin, K. Witte, A., Reisinger, G., Lubitz, W. and Basi, U. (1995). Evaluation of the *E. coli* ribosomal rnbB P1 promoter and phage derived lysis genes for the use in biological containment system: a concept study. *J. Biotechnol.* **39**, 137 – 148.

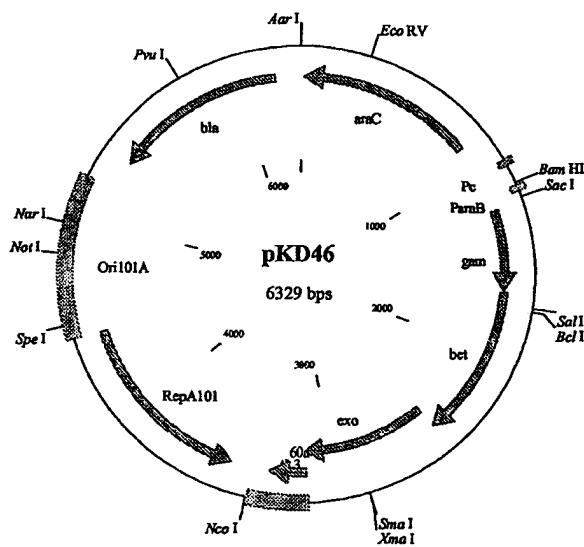
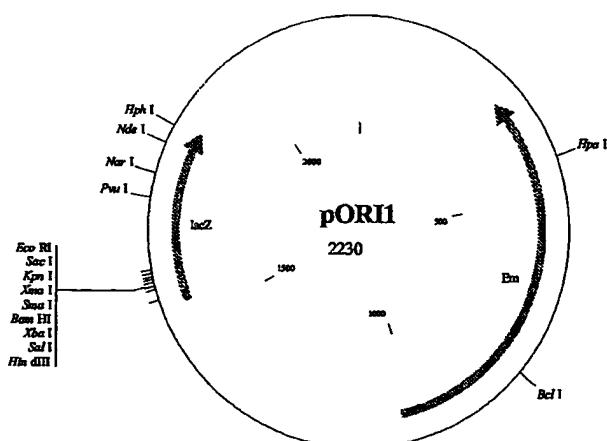
5

Claims

1. An isolated strain of *Lactobacillus* sp. carrying a mutant *thyA* gene, whereby said gene is inactivated by gene disruption.
2. An isolated strain of *Lactobacillus* sp. according to claim 1, whereby said *Lactobacillus* sp. is *Lactobacillus salivarius*.
3. An isolated strain of *Lactobacillus* sp. according to claim 1, whereby said *Lactobacillus* sp. is *Lactobacillus plantarum*.
4. The use of a strain of *Lactobacillus* sp. according to any of the claims 1-3 as host strain for transformation, whereby the transforming plasmid does not comprise an intact thymidylate synthase gene.
5. An isolated strain of *Lactobacillus* sp. according to any of the claims 1-4, comprising a transforming plasmid that does not comprise an intact thymidylate synthase gene.
6. An isolated strain of *Lactobacillus* sp. according to any of the claims 1-5 comprising a gene encoding a prophylactic and/or therapeutic molecule.
7. An isolated strain of *Lactobacillus* sp. according to claim 6 wherein said prophylactic and/or therapeutic molecule is interleukin-10.
8. The use of an isolated strain of *Lactobacillus* sp. according to claim 6 or 7 for the delivery of prophylactic and/or therapeutic molecules.
9. A pharmaceutical composition comprising an isolated strain of *Lactobacillus* sp. according to the claims 6 or 7.
10. The use of an isolated strain of *Lactobacillus* sp. according to claim 6 or 7 for the preparation of a medicament.
11. The use of an isolated strain of *Lactobacillus* sp. according to claim 6 or 7 for the preparation of a medicament to treat inflammatory bowel diseases.

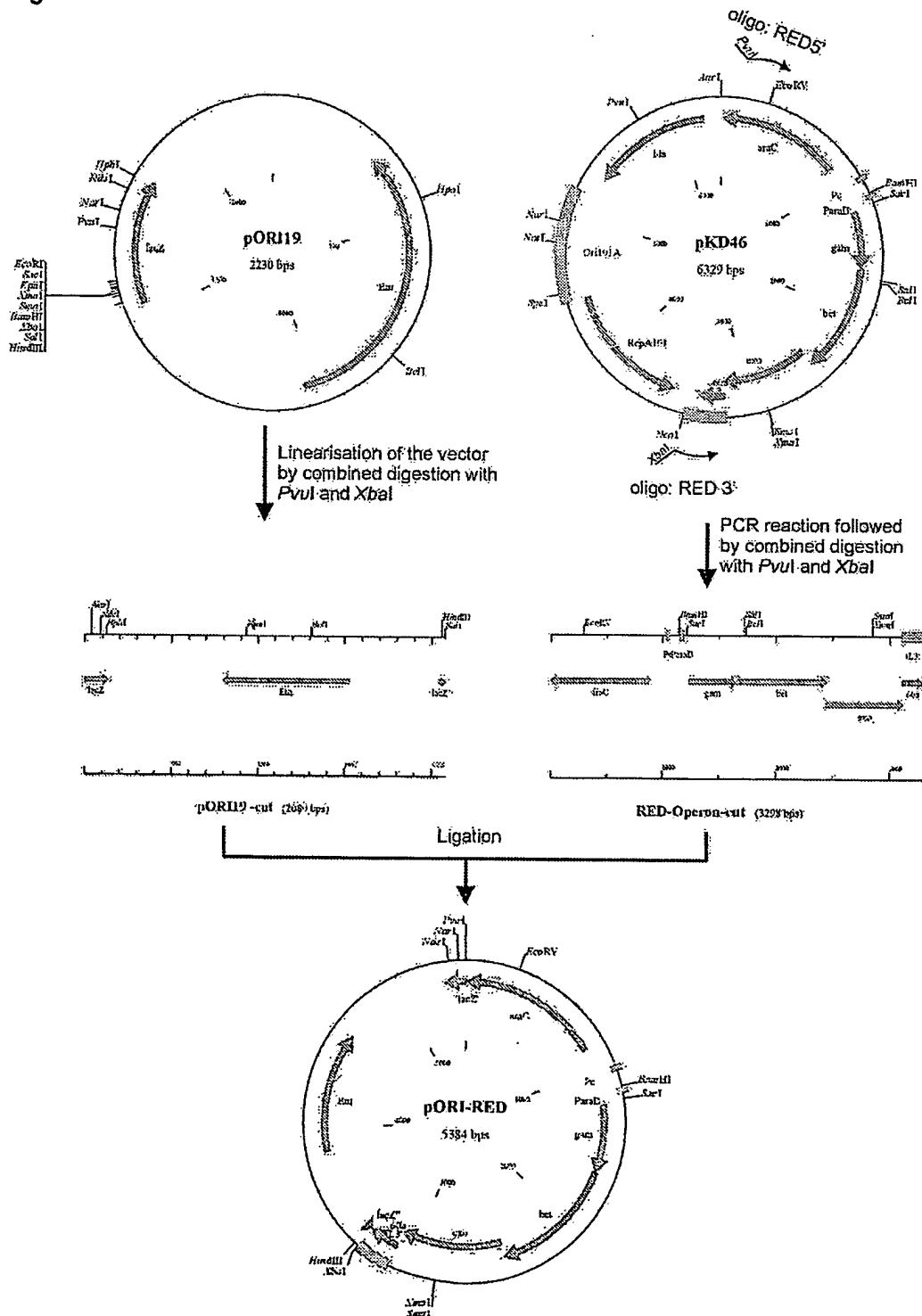
5

1/3

Figure 1:**Figure 2:**

2/3

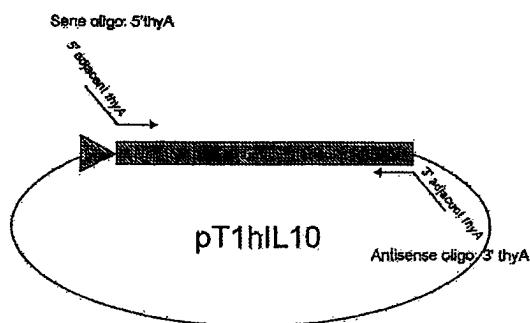
Figure 3:



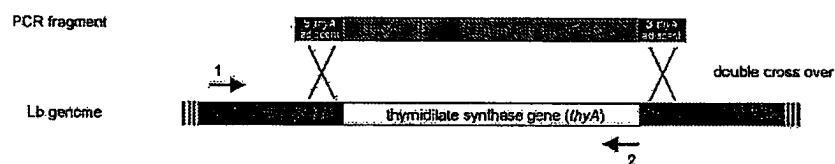
3/3

Figure 4:

STEP 1: PCR amplification of the *hil-10* gene flanked by the adjacent 5' and 3' *thyA* region



STEP 2: Electroporation of the STEP 1 PCR fragment in the Lactobacillus strain that expresses the lambda Red recombinases by the plasmids pVE6007 and pORI-RED, causes gene-replacement of the *thyA* gene by the *hil-10*.



STEP 3: Successful gene replacement of the genomic Lactobacillus *thyA* gene by the *hil-10* secreting cassette.

